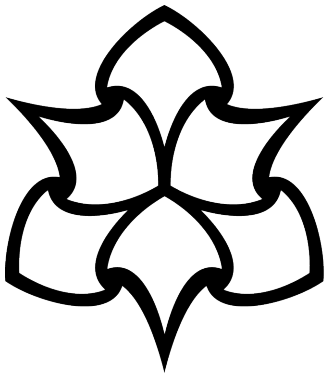

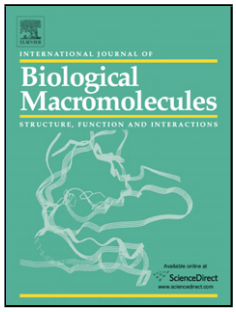

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ATLAS: an Advanced PCR-Method for Routine Visualization of Telomere Length in *Saccharomyces cerevisiae*

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Research highlights:

- a robust PCR methodology was developed and fully verified for amplification of terminal telomeric DNA at six chromosomal locations in budding yeast;
- a new approach was proposed for designing reverse telomeric primers based on the ligation of an adaptor of a fixed size;
- the method could be used for routine analysis of telomere length and sequencing telomeric DNA.

A B S T R A C T

Measuring telomere length is essential in telomere biology. Southern blot hybridization is the predominant method for measuring telomere length in the genetic model *Saccharomyces cerevisiae.* We have further developed and refined a telomere PCR approach, which was rarely used previously (mainly in specific telomeric projects), into a robust method allowing direct visualisation of telomere length differences in routine experiments with *S. cerevisiae*, and showing a strong correlation of results with data obtained by Southern blot hybridization. In this expanded method denoted as ATLAS (A-dvanced T-elomere L-ength A-nalysis in *S. cerevisiae*), we have introduced: 1) a set of new primers annealing with high specificity to telomeric regions on five different chromosomes; 2) a new approach for designing reverse telomere primers that is based on the ligation of an adaptor of a fixed size to telomeric ends. ATLAS can be used at the scale of individual assays and high-throughput approaches. This simple, time/cost-effective and reproducible methodology will complement Southern blot hybridization and facilitate further progress in telomere research.

1. Introduction

Ends of nuclear eukaryotic chromosomes possess telomeres that are structures containing repetitive DNA (telomeric DNA) and specific proteins. Telomeres are essential for complete replication and genetic stability of chromosomes [1]. Telomere length (TL) determined by the length of telomeric DNA is a molecular indicator of cellular senescence and aging in higher eukaryotes including humans [2-4]. TL is also associated with lifespan [5-8]. Telomere dysregulation causes genetic instability and cancer [9-11], a number of degenerative disorders [12] and vascular senescence [13]. This area of research involving humans was remarkably facilitated when a "gold standard" procedure for measuring TL based on Southern blot hybridization (SBH) [14, 15] was complemented by real time quantitative PCR (qPCR) [16]. The qPCR, however, does not allow direct determination of TL (which is an advantage of SBH).

Many aspects of telomere biology are studied using a premier eukaryotic model *Saccharomyces cerevisiae* (budding yeast) [17-19]. In this species, TL is controlled genetically by at least 272 non-essential and 87 essential single genes [20, 21]. A complex gene network of about 400 genes (including mentioned above 359 genes) involved in controlling TL in yeast via gene interactions has been identified [22]. Recent implications of different environmental and stress factors and drugs into telomere attrition [23-25] indicate that the scope of TL regulation by extrinsic factors in yeast is considerably broader than was previously thought. This expanding area of research requires an efficient and convenient methodology for rapid screening of TL [17].

Presently, SBH is the preferred method for determining TL in *S. cerevisiae*, however, this reliable technique requires a relatively high degree of skills and is time-consuming. Routine PCR approaches do not work for TL analysis in budding yeast because of irregularity in yeast telomeric DNA repeats [26] preventing canonical design of specific reverse primers. The very ends of *S. cerevisiae* telomeric DNA are composed of heterogeneous in size GC-reach $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats. Their total size in different WT strains of *S. cerevisiae* (depending on genetic background) varies between 100 and 500 bp, with modal size 300-350 bp. The loss of these repeats results in high rate of loss of correspondent chromosomes [4, 18, 27]. There are two classes of subtelomeric sequences adjacent to $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats: up to four Y'-elements (proximal to $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats) and one X-element ($\geq$ 0.5 kb) proximal to Y'-sequences. In fact, a little proportion of $C_{1\text{-}3}A/TG_{1\text{-}3}$ sequences exists between X-element and Y'-elements, which makes Y'-sequences embedded within $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats. X- and Y'-repeats contain autonomously replicating sequences. Y'-elements could be 5.2 kb or 6.7 kb, with the total length up to 25 kb; they often encode helicases and present only in about half of chromosomal ends. X-elements are present in all telomeres [18, 27, 28]. The repetitive nature of all telomeric regions makes difficult designing primers for PCR amplification of individual telomeric DNA fragments correlating with TL. Nevertheless, although limited for a general practical use, a possibility of PCR amplification of telomeres in a *S. cerevisiae* model strain (with genetic insertion of a unique *ADE*2-marker at the right arm of chromosome V) was demonstrated by using terminal transferase-mediated C-tailing of chromosomal ends [29]. The insertion of the *ADE*2-marker allowed designing a unique forward primer annealing not far from the end of chromosome V, and C-tailed ends were suitable for designing poly-G reverse primers, thus making possible amplification of a unique PCR fragment from the telomeric end.

The approach with *ADE2*-marked chromosome VR has been used in a few studies on the fine structure of telomeric repeats in wild type and mutant strains of *S. cerevisiae* [30-35]. Similarly, telomere DNA has been amplified from *URA3*-marked chromosome VIIL [36]. The integration of artificial genetic markers is the main limitation for the usage of telomere PCR in non-marked yeast

strains. A few research groups have aimed to avoid the integration of artificial constructs near telomeric DNA and utilised forward primers annealing to residential sub-telomeric sequences on individual chromosomes of *S. cerevisiae*: IL [30, 34, 37-44], VIR [35, 42, 43, 45], VIIL [43, 46], XIVR [42] and XVL [45]. Yet other experiments have been designed to target Y' sequences localised on different chromosomes [47, 48]. These studies have demonstrated the productivity of the telomere PCR approach without genetic markers at telomeres for clarification of fundamental aspects of telomere metabolism in budding yeast cells compromised in different genetic functions. However, this time- and cost-effective approach is still not widely used for trivial analyses of TL in numerous projects aiming the investigation of different genetic and environmental factors in TL regulation using SBH [17, 18, 20, 23, 24]. The limited use of telomere PCR for direct TL measurements is possibly due to the robustness of telomere SBH technique and due to potential technical problems associated with some published primers that have been used for other applications rather than for direct visualisation of TL. The telomere PCR was recently adapted for analysis of TL on chromosome IIL in *Aspergillus nidulans* [49]. Here we present an extended and refined telomere PCR methodology to provide a convenient and simple tool for routine direct analyses of TL in various strains of budding yeast. This method denoted as ATLAS (A-dvanced T-elomere L-ength A-nalysis in *S. cerevisiae*) allows analysis of TL in S. cerevisiae on six new telomeric regions including those on three chromosomes that have not been analysed previously. We further propose an alternative approach for designing reverse primers based on the ligation of a synthetic adaptor to the end of telomeric sequences. The method involves restriction enzyme digestion of certain telomeric amplicons of large size for enhancing sensitivity of TL analysis, if necessary. Our approach allows extended options for detailed molecular analysis of telomeric DNA on a range of chromosomes and could complement the SBH technique for a routine examination of TL in *S. cerevisiae* in many laboratories.

2. Materials and methods

2.1. Yeast strains and culture conditions

Wild-type and mutant strains of *S. cerevisiae* with normal, short and long telomeres (**Table 1**) were grown for 2-3 days at 23ºC on solid or overnight in liquid YEPD medium [50] supplemented with adenine sulphate. For gradual telomere shortening by elevated temperature, wild-type strains were passaged up to 12 passages at 36ºC.

2.2. Isolation of DNA

Three simple protocols were used for isolation of total DNA in order to test their suitability for telomere PCR. They all gave comparable results.

Protocol 1. The Yale protocol adopted in the lab of D. Lydall (Newcastle University) was used with some modifications. Pellets of cells collected in 2 ml tubes were resuspended in 250 µl of the solution containing 0.1 M EDTA (pH 7.5), 14 mM β-mercaptoethanol and 2.5 mg/ml of Zymolyase 20T (Seikagaku Biobusiness Co.) and spheroplasted by incubating at 37ºC for 1hour. 50 µl of the miniprep mix (0.25 M EDTA (pH 8.5), 0.5 M trisbase, 2.5% (w/v) SDS) was added to each tube with the following incubation of the mixture at 65ºC for 30 minutes. After lysis, 63 µl of 5 M potassium acetate was added to each sample, and tubes were incubated on ice for 30 minutes. After spinning the samples at full centrifuge speed for 20 minutes, supernatants with DNA were transferred to 1.5 ml tubes containing 720 µl of 100% ethanol and centrifuged for 5 minutes. Each pellet was re-suspended to homogenous state in 130 µl of 1xTE buffer containing 1mg/ml of RNA-ase A (Qiagen) and incubated at 37ºC for 35 minutes. DNA was precipitated by adding 130 µl of isopropanol and centrifugation for 10 min. The pellets of DNA were washed briefly in 0.5 ml of 70% (v/v) ethanol followed by spinning for 1 min. After complete aspiration of ethanol, DNA pellets were air dried and dissolved in 30 - 100 µl of water (or 1xTE buffer for long storage) and stored at -20ºC.

Protocol 2. DNA was isolated as in *Protocol 1* but the procedure included the step of purification by phenol:chloroform:isoamyl alcohol extraction (12:12:1, v/v/v) after the stage of treatment with RNA-ase A.

Protocol 3. A very quick DNA isolation procedure was based on the protocol recommended by the manufacturer of the Bioline Isolate II Genomic DNA kit. Total DNA was eluted from the column by adding 100 µl of pre-heated to 70ºC elution buffer.

2.3. Southern blot hybridization

Total DNA samples (1-2 µg) were cut with XhoI and electrophoresed in 0.8% agarose (0.5 x TBE buffer) at 25V overnight. Gels were soaked twice (10 min each time) in 0.25M HCl, with gentle shaking. After rinsing quickly in water, the gels were soaked twice (for 10 min) in the solution containing 1.5M NaCl and 0.5M NaOH, with gentle agitation. After a brief washing, the gels were neutralized by shaking in 1M ammonium acetate (twice for 10 min) and transferred to Hybond^{TM}-N+ membrane (GE Healthcare Limited) overnight. DNA was bound to the membrane by UV cross-linking (0.2 J/cm^2). The hybridization of filters with a Y' + TG probe [51] [or Chr VI-specific probe] and detection of the hybridization signals were performed according to the booklet of Amersham for Gene Images Alkphos Direct Labelling and Detection System (GE Healthcare Limited).

2.4. Terminal transferase mediated tailing of genomic DNA

This procedure is a generalised protocol based on published literature. About 200 ng of genomic DNA (estimated from stained agarose gels) in a volume of 10 µl were denatured at 95 °C for 5 min in a thermal cycler and cooled on ice. The DNA was then spun down for a few seconds in a microcentrifuge to collect the drops, and 40 µl of transferase reaction mix was added to make the total volume 50 µl.. The transferase reaction mix contained 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 0.25 mM $CoCl_2$, 0.2 mM dCTP and 2U of terminal transferase (New England Biolabs). The mix was incubated at 37 °C for 30 min, and then enzyme was inactivated by heating the samples for 10 min at 70 °C. After heat inactivation, the tailing reactions were used in PCR.

2.5. PCR for amplification of terminal chromosomal ends

PCRs were performed on tailed DNA in 20 µl using GoTaq® Long PCR master Mix. PCR mix included 10 µl of GoTaq® Long PCR master Mix, 0.5 µM of a chromosome specific primer, 1 µM of a telomeric primer (**Table 2**) and 1-3 µl of C-tailed genomic DNA. PCR conditions (**Table 3**) were worked out and optimised for each set of primers. PCR reactions were carried out in 0.2 ml tubes (Thermo-Tube, ABgene) using Quanta Biotech Q-Cycler II. No template controls (NTC) were included in each set of PCR.

2.6. Ligation of the adaptor to telomeric ends

Adaptor ligation was performed as previously described [52], with some modifications. The sequence of the anchor oligonucleotide was

5'- PHO-TTTAGTGAGGGTTAATAAGCGGCCGCGTCG-ddC3' (Sigma). It contained phosphate at the 5' end and 2',3'-dideoxycytidine (ddC) at the 3'end. The ligation reaction was performed overnight (for the convenience reason) at room temperature in total volume of 25 µl and contained: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1mM ATP, 20% (w/v) PEG8000, 1 mM hexamine cobalt chloride, 1 µl T4 RNA ligase (20U/µl, Thermo Scientific), 1.3 µM Adaptor and ~ 300 ng of genomic DNA. Alternatively the ligation reaction could be carried out during 1-2 hrs (according to the manufacturer's guidelines). Ligation reactions were terminated by heating at 70° for 10 min.

To remove the unused adaptor molecules from the ligation mix, DNA was precipitated. For the precipitation, TE buffer was added to the ligation mix to make a final volume of 200 µl. 100 µl of 30% (w/v) PEG8000/30 mM $MgCl_2$ was added to the ligation mix and mixed thoroughly. DNA pellet was collected after 20 min of centrifugation at 10,000 rgf at room temperature and dissolved in 20 µl of sterile ddH_2O.

2.7. PCR from the adaptor-ligated DNA

PCR was performed in a total volume of 15 µl and contained: 1.5 µl of 10xPCR buffer, 0.3 µl of 10 mM dNTPs, 0.2 µl of 33 µM chromosome specific primer, 0.3 µl of 33 µM r-ad2 primer, 0.3µl of HotstarTaq DNA Polymerase (Qiagen) and 2 µl of DNA precipitated from the ligation mix.

PCR conditions were the following:

95°C – 15 min, and then 42 cycles at 94°C – 30 sec, 67°C – 30 sec, 72°C -1 min 30 sec with final extension at 72°C for 10 min. Samples with NTC were included in each PCR.

2.8. Cloning and sequencing of telomeric DNA

PCR fragments were cut out of agarose gels, purified on the columns (Qiagen) and cloned into pGEM®-T Easy Vector (Promega). Plasmid DNAs were isolated from overnight liquid cultures of individual transformants and analysed for the presence of inserts by digestion with EcoRI. DNA from positive clones has been sequenced using GATC Biotech sequencing facilities.

3. Results and discussion

The aim of this research was to expand existing telomere PCR methodology in order to improve it, and to emphasise possibilities of using it for routine analysis of TL in budding yeast by direct visualisation of PCR products from individual chromosomes. Thus, we developed novel primers and provided the experimental evidence for correlations of data on TL obtained by SBH and by ATLAS using the same sets of wild type and mutant strains with normal, short and long telomeres (Table 1). In addition, we propose an alternative to poly-C tailing approach for making telomere DNA end suitable for designing reverse primers. This approach is based on the ligation of a fixed size synthetic adaptor to telomeric repeats.

A few forward primers described in the literature for amplification of telomeric DNA anneal to chromosomes IL, VIR, VIIL, XIVR and XVL (see references in the Introduction). Some primers have been designed for specific aims and cannot be robust for routine analyses of TL in other strains. We found that some of the primers anneal to more than one locus/chromosome. For example, the primer for VIIL chromosome [53] has 100% homology also to chromosomes III, VIII, XIV, XV and XVI in S288C background. Gels with actual amplified fragments were presented only in a few papers. Clear bands suitable for comparison of TL were shown for chromosomes IL [37], VIR [35, 48] and XVL [54]. The interpretation of visual differences in TL from some published gels was complicated because of: a) large variation in size of fragments amplified from chromosome VIIL and small differences in TL [55]; b) not pronounced TL differences in the used strains after amplification of telomere regions from

chromosomes VIR and XVL [54]. The majority of authors did not show images of PCR products, possibly because the described primers were used for cloning and sequencing rather than for the visualisation of TL. The primers used for ADH4 locus on Chr VIIL [55-57] are not likely to be robust for TL visualisation because they anneal very far from the end (-16 kb).

We have designed forward primers (Figure 1; Table 2) that anneal within six new chromosome-specific sub-telomeric DNA regions located at different distance (1.25-5.23 kb, according to the *Saccharomyces* Genome Database [SGD]) from the ends of chromosomes IR, IIR, VIR, IXR and XIR. Two primers for VIR allow either short or long amplicons (Table 3). Every forward primer presents a sequence for preferential annealing on a telomeric region of a single chromosome. Three modifications of reverse primers (universal for all chromosomes) contained poly-G oligonucleotides complementary to telomeric DNA C-tail generated by terminal transferase (Figure 1; Table 2). Amplicons from different chromosomes are variable in size because of different minimal distances between chromosomal ends and available distal regions that we were able to locate in order to design unique, specific for a chromosome forward primers. According to SGD, the size range for amplicons from published telomeric primers varies from less than 100 bp [35, 37, 46] to ~ 16 kb [46, 55]. Thus, overall we attempted to reduce the range of amplicon size to minimize technical problems of PCR and to aid visual detection of TL. For the amplification of large fragments, we used a Taq polymerase for amplifying long targets.

All six primer sets designed for five chromosomes revealed reliable direct visualisation of TL differences in wild type *S. cerevisiae* and well-studied mutant strains with short and long telomeres (Figures 2-4, 6; Supplementary Figures 1-3). Short telomeres of the mutants resulted from deletions of *MRE11* and *YKU70* while long and very long telomeres were caused by deletions of *PIF1* and *RIF2* genes [18, 20]. The TL results obtained by ATLAS strongly correlated with those obtained for the same mutants by SBH method (Figures 2a, 4, 5; Supplementary Figure 1), suggesting that ATLAS is reproducible and accurate method (Supplementary Note 1). The lengths of telomeric fragments amplified by PCR from different chromosomes in wild type strains were slightly larger than values predicted according to SGD. This is possibly because telomeric sequences (for S288C strain) available from SGD do not necessary include the full length of telomeric repeats, due to the uncontrolled integration of artificial inserts used for sequencing of chromosomal ends [26, 58]. Consistent with this, the differences between predicted and actual sizes of telomeric amplicons could be found in published research [35, 37].

The telomeric DNA fragments amplified from chromosomes I, II and VI (with predicted sizes 3.64 kb, 2.83 kb and 5.23 kb respectively) could be digested by appropriate restriction enzymes at the correspondent restriction sites that are available in sequences of these fragments (Figure 2). This allows

fine alignments of variable in size small bands (containing terminal repeats) against the larger sub-telomeric DNA bands of the same size (Figure 2d; Supplementary Figure 1b). The aligned bands enhanced visual resolution between different TLs by forming a straight line serving as a control for the migration of analysed bands in a gel. We suggest that this is an optional step that could increase the sensitivity of the method when it is necessary, for example, when the differences in TL are subtle, especially in long amplicons for particular chromosomes (Supplementary Note 1**)**. Moreover, the step of digestion was a simple and useful approach for us to start proving the telomeric nature of the amplicons (at least from some chromosomes), based on the expected sizes of smaller telomeric fragments that were easy to see on gels even after short time of electrophoresis. The presence of a large fragment (of the same size predictable from SGD) and a smaller fragment (with size depending on TL) allows quite accurate detection of the difference between their length in each individual lane of a gel, thus providing a useful visual criterion for differences in TL in samples from different lanes. A similar idea on an additional (internal to each lane on a gel) marker is often used in SBH experiments. It is especially helpful when using gels with multiple lanes [24] that are sometimes deformed during electrophoresis, and the presence of MW ladders even on both sides of a gel is not always effective for precise detection of fragment sizes.

Mutants *yku70*Δ have shortened telomeres [59] but additional deletion of *EXO1* partially compensates the shortening [60]. The subtle difference in TL between *yku70*Δ and *yku70*Δ *exo1*Δ mutants was resolved by SBH using a Y'-probe and a Chr VI specific probe [60]. We have convincingly reproduced this with ATLAS using primers for two chromosomes, Chr VI (data not shown) and ChrXI (Figure 4). Similarly, *exo1*Δ *cdc13-1* mutant has slightly longer telomeres than WT that is detectable by SBH and reproduced by ATLAS (Supplementary Figure 1). Recent studies showed that chronic exposure to different environmental factors could affect TL in budding yeast independently on genetic de-regulation [23, 24]. Figure 5 (top panel) shows the dynamics of gradual telomere shortening (detected by SBH) after exposure of wild type *S. cerevisiae* cells to elevated temperature (36^{o}C) during 12 consecutive passages on YEPD medium. The analysis of the same DNAs by ATLAS revealed the same trend of telomere shortening, with sufficient resolution of differences in TL for each DNA (Figure 5, bottom panel). Thus, our results suggest that ATLAS is a sensitive method for detecting even minor differences in TL (Supplementary Note 1). Differences between TL in mutants *pif1*Δ *exo1*Δ and *rif2*Δ (both with long telomeres) were also resolved sufficiently by ATLAS (Supplementary Figure 3). Very long telomeres known for *rif1*Δ and *rif2*Δ mutants [20] were also well distinguished from each other (Figure 6), thus indicating the applicability of the method for discriminating TL in a wide range of sizes.

Bands of telomeric amplicons are always wider than bands of DNA ladders. It could be interpreted in terms of heterogeneity of telomeric DNA taking place even for the same chromosomes in different cells.

The bands amplified from long telomeres are more smeared than those from shorter telomeres (especially pronounced after longer electrophoresis, Figure 6 and Supplementary Figure 2), suggesting more heterogeneous sizes of telomeric DNA in long telomeres. Although the range of mutants we have used did not cause problems in TL detection, the potential lack of signals is not e
xcluded in some rare scenarios involving long, heterogeneous telomeres.

In some cases, using terminal transferase reaction for the extension of telomeric ends could result in C-tails of different size in different samples, especially in samples from different experiments. For example, different lengths of C-tails were shown in cloned telomere PCR sequences of *A. nidulans* and discussed as possible reasons contributing to smeary PCR products [49]. The nature and conditions of this reaction, in particular, if it is carried out in independent assays, imply possibilities for variable lengthening. In the *A. nidulans* study, using "G-only" primers did not result in amplifying specific telomeric products at all, and sufficient specificity was achieved by designing advanced "telomere-anchored primers" based on the addition of 6-nucleotide telomeric sequences upstream poly-G [49]. This suggests that at least for some chromosomes of this species C-tailing approach might be not effective enough in terms of specificity. Although we did not come across such clearly proved cases in budding yeast, it is not excluded, however, that the non-specificity of poly-G primers might be a limitation for amplifying telomeric repeats from certain chromosomes also in *S. cerevisiae*. Thinking prospectively on how to exclude size variation in extended telomeric ends, to minimize even potential non-specificity of poly-G primers, and adhering to a general idea of designing primers preferably without excessively repeated nucleotides, we propose another alternative to C-tailing approach that is based on the ligation of an adaptor of a fixed size to the chromosomal ends. We used the principle of the adaptor described by [52]. The adaptor (see Materials and Methods) is a 30 bp oligonucleotide containing phosphate on 5'-end and 2',3'-dideoxycytidine at 3'-end. These modifications allow the ligation of only one adaptor molecule to each chromosome end, thus resulting in the uniform size of the extensions on all chromosomes. The DNA with the ligated adaptor (see Materials and Methods) served as a template for PCR amplification using the same forward primers and reverse primer r-ad2 annealing to the adaptor (Table 2). The TL analysis by this method performed with forward primers for two chromosomes (VIR and XIR) had similar efficiency as in experiments with C-tailed templates (Figure 7). Using the adaptor allows the amplification of the uniform size telomeric fragments from the same template, thus allowing comparisons of results from different experiments. This could be a potentially useful feature in: a) wide-genome TL studies involving numerous experiments at different times, b) cases where unpredictable peculiarities of telomeric structures in different mutants might create concerns regarding the uniformity of C-tails, and c) where small differences in TL need to be detected. The successful amplification of telomeric DNA with the adaptor also implies the possibility to use any

other sequences as adaptors – in case if "genetically modified" telomeric amplicons are required for any other projects beyond TL analyses.

Although products of telomeric PCR were repeatedly proved to be telomeric DNA in the previous studies with other primers, we wanted to approve the same for our primers and conditions (in particular, because of many cycles recommended for PCR reactions). To confirm telomeric DNA identities in PCR fragments amplified from C-tailed and ligation-anchored templates, we cloned and sequenced PCR products for normal size telomeres (in wild type cells) and short telomeres (in mutants). Sequencing results showed that the cloned DNA fragments contain telomeric DNA repeats of sizes expected for normal and short telomeres (Figure 8). The clones also contained expected sequences of C-tails and the adaptor at the very end (Supplementary Figure 4).

ATLAS is an expanded and improved PCR-based method that can be used for repeatable, fast and accurate visualisation of TL in *S. cerevisiae*, with high specificity of primers to five chromosomes including three (II, IX and XI) that have not been used in TL studies before. We have designed primers for S288C genetic background with 100% homology to the correspondent telomeric regions in W303 background. For the first time in the practice of using telomere PCR for budding yeast, we demonstrate that there is a strong correlation of quantitative TL data obtained by SBH and PCR for strains with normal, short and long telomeres (Figure 9). The procedures described here were repeatedly tested and unified for a broader range of primers than it was published in any other studies up to date. These protocols were repeatedly reproducible in the course of our experimentation.

As a complementary to SBH alternative in routine TL studies, ATLAS has some advantages over SBH (Supplementary Note 2). ATLAS is faster and simpler than terminal restriction fragments (TRF) analysis by SBH. It requires much less DNA that could be isolated easily by using simple protocols. As a cost-effective method with mild demands for technical training, it provides extended choices for a wide range of researches aiming either routine studies of TL or more complex aspects of yeast telomere biology. The ATLAS results obtained for all analysed chromosomes by using undigested and digested with restriction enzymes amplicons were consistent in reflecting TL differences detected by SBH. This implies that any particular assay described here could be effectively used for analyses of *S. cerevisiae* TL (Supplementary Note 1). ATLAS allows analysis of TL alterations specific to a few individual chromosomes, with possible cloning and sequencing of amplified fragments to determine precisely all changes in X regions of telomeric DNA as well as the actual size of X-telomeric DNA that is not predictable from SGD. Although the method is based on analysis of X-telomeric regions, the strong correlation with data from TRF, which includes analysis of X- and Y'-sequences, it provides a reliable tool for TL analysis suitable for different applications. The method allows also easy generation of telomeric probes for telomere analyses by SBH when necessary. The efficiency and simplicity in

handling allow scaled approaches in using ATLAS according to criteria for high throughput techniques (Supplementary Note 3). It could be suitable for large-scale screening for factors affecting TL, including new genetic interactions as well as a wide range of physiological and environmental stimuli [17, 24].

4. Conclusions

This research offers an advanced tool set (ATLAS) for direct reproducible PCR analysis of TL in *S. cerevisiae* strains with short and long telomeres. It includes primers for amplification of telomeric DNA repeats in six new chromosomal locations and a new approach for designing reverse primers based on the ligation of the adaptor to chromosomal ends. Sequencing of cloned PCR fragments revealed that they contain telomeric DNA. TL determined by ATLAS strongly correlated with TL calculated from SBH experiments. These data suggest that ATLAS will be a useful and quick method that could complement SBH in many occasions where routine but extensive TL measurements are needed, in particular, for wide-genome TL screens as well as for screening multiple environmental and metabolic factors that deregulate TL in budding yeast. Moreover, ATLAS will expand possibilities for cloning and sequencing telomere repeats from new chromosomal loci.

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Appendix A. Supplementary data

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FIGURES

Figure 1. The location of primers for amplification of telomeric DNA by ATLAS PCR from five chromosomes of *S. cerevisiae*. DNA ends of all chromosomes are C-tailed by terminal transferase (**A**) or extended by the ligation of the adaptor (**B**). Larger amplicons from three chromosomes contain unique restriction sites allowing additional options to analyse the lengths of shorter TRFs after digestion of PCR products with the restriction enzymes. The sizes of the TRFs after digestion are shown in bold (they were calculated according to the information in SGD for genetic background S288C of *S. cerevisiae*). A generic scheme (**B**) shows the ligation of the adaptor to chromosomes and the reverse primer that can be used with any chromosome-specific forward primers. Chr – chromosome; RA – right arm.

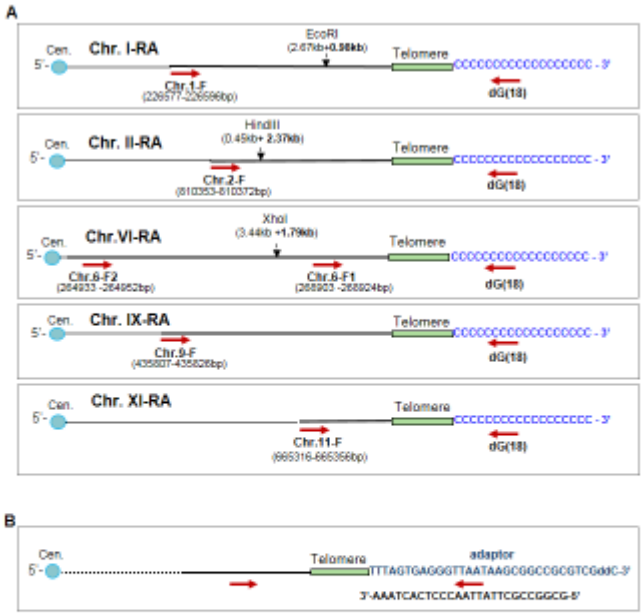

Figure 2. The correlation of results on genetic telomere length deregulation obtained by TRF (SBH method) and by ATLAS. **(A)** Different telomere length in wild type and deletion mutants confirmed by TRF: 1,2 – DLY1479 (*mre11*Δ), 3,4 – DLY1331 (*mre11*Δ), 5,6 - DLY640 (WT), 7,8 – DLY641 (WT), 9,10 – DLY5395 (*pif1*Δ *exo1*Δ), 11,12 – DLY5396 (*pif1*Δ *exo1*Δ). **(B)** Detection of short, normal and long telomeres in the same mutants by ATLAS (primers for Chr VI-RA; forward primer Chr6-F1 for the small amplicon): 1 - DLY1331 (*mre11*Δ), 2 – DLY641 (WT), 3 – DLY5395 (*pif1*Δ *exo1*Δ), 4 – NTC. **(C)** ATLAS assays (primers for Chr XI-RA): 1- DLY 1331 (*mre11*Δ), 2 – DLY 640 (WT), 3 –DLY 5395 (*pif1*Δ *exo1*Δ), 4 – NTC. **(D)** Direct and inverted gel images for ATLAS PCR products (primers for Chr VI-RA) digested with *Xho*I: 1- DLY 1331 (*mre11*Δ), 2 – DLY 1481 (*mre11*Δ), 3 – DLY 1479 (*mre11*Δ), 4 - DLY 640 (WT), 5 – DLY 641 (WT), 6 – DLY 1296 (*exo1*Δ *cdc13-1*), 7 – DLY 5395 (*pif1*Δ *exo1*Δ), 8 – DLY 5396 (*pif1*Δ *exo1*Δ). Forward primer was Chr6-F2 (for the large amplicon). DNA MW markers: (**A**) - 1 kb STEP LADDER (Promega), (**B-D**) - 1 kb plus (Thermo Scientific).

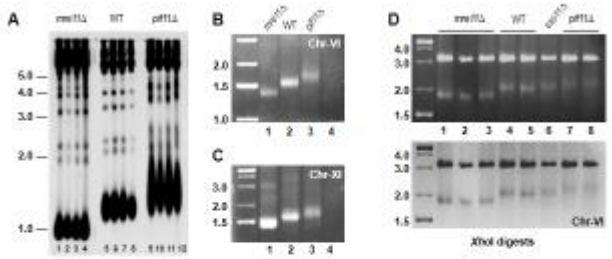

Figure 3. Reproducibility in detecting TL differences by ATLAS using primers for long amplicons from different chromosomes. The efficiency of detecting normal, short and long telomeres by ATLAS was compared after amplification of variously sized fragments from three different chromosomes (indicated at the bottom of the figure). The same sets of C-tailed DNAs were used as templates for PCR reactions to amplify telomeric DNA: 1, 5, 9 – NTC, 2, 6, 10 – DLY641 (WT), 3, 7, 11 - DLY1479 (*mre11*Δ), 4, 8, 12 – DLY5395 (*pif1*Δ *exo1*Δ). MW marker: 1 kb plus (Invitrogen). Conditions for electrophoresis: long (20 cm) 0.8% agarose gel, 0.5 x TBE, 80v (for ~5 hrs).

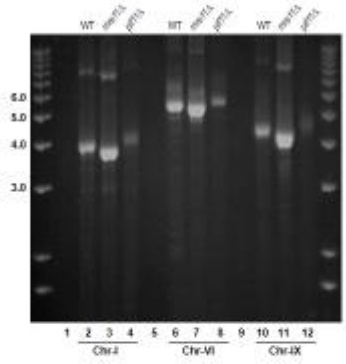

Figure 4. Reproducibility in detecting a subtle TL difference by SBH (top) and ATLAS (bottom; primers for Chr XI-RA). The same DNAs were subjected to analyses of TL by both methods: 1 – DLY 640, 2 - DLY 1412 (*yku70*Δ), 3 – DLY 1408 (*yku70*Δ *exo1*Δ). For TRF, XhoI digest was used. MW markers in PCR: 1 kb plus (Thermo Scientific). MW markers in SBH: 1 kb ladder (Invitrogen).

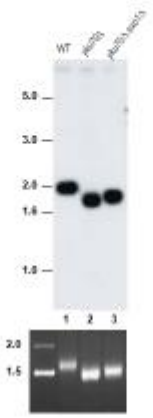

Figure 5. High temperature-induced telomere shortening effects detected by two methods. Wild type cells (DLY640) were sub-cultured at 23°C and 36°C: 1- 23°C, 4 passages; 2 - 36°C, 3 passages; 3 – 36°C, 6 passages; 4 - 36°C, 9 passages; 5 – 36°C, 12 passages. Isolated DNAs were analysed for TL by TRF (top) and by ATLAS for Chr. XI-RA (bottom). MW markers in PCR: 1 kb plus (Thermo Scientific). MW markers in SBN: 1 kb ladder (Invitrogen).

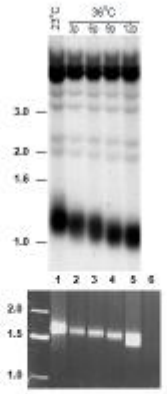

Figure 6. Resolution of differences in TL in strains with long and very long telomeres. The reverse primer dG(18) and forward primer for Chr XI-RA were used. 1 – YCR36 [*RIF1*$^{+}$ *RIF2*$^{+}$], 2 – YCR116 (*rif2*Δ), 3 – YCR272 (*rif1*Δ). MW markers: 1 kb plus ladder (Invitrogen).

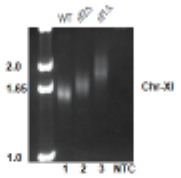

Figure 7. Detection of TL by using templates with the ligated adaptor. The reverse primer p-Ad (for the adaptor) and forward primers for Chr VI-RA (**A**) and Chr-XI-RA (**B**) were used. 1 – DLY1479 (*mre11*Δ), 2 – DLY641 (WT), 3 – DLY5396 (*pif1*Δ *exo1*Δ). MW marker: 1 kb plus ladder (Invitrogen).

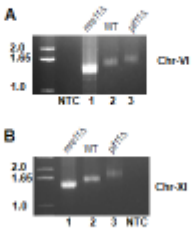

Figure 8. Sequences of cloned telomere PCR products amplified from the chromosome XI-RA. The sequences are shown for mutants with short telomeres (clones1-4) and for wild type with normal telomere length (two independent clones, 5-6). The sequence from X-element combinatorial repeat near telomeric repeats (shown in bold) is consistent with SGD data for Chr 11R.

>clone1 (122 bp), DLY 1479
TAGGGTAGTGTTAGGGTTGTGGGTGTGTGGGTGTGGTGTGGGTGTGGGTGTGGTGTGGGTGTGGTGTGGGTG
TGGTGTGGGTGTGTGGGTGTGGTGTGTGGGTGTGTGGTGTGTGGGTGTGGGTGTGGTGTGGG

>clone2 (77bp), DLY 1479
TAGGGTAGTGTTAGGGTTGTGGGTGTGTGGGTGTGGTGTGGGTGTGGGTGTGGTGTGTGGGTGTGGTGTGTGT
GGGTGTGGTGTGTGGGTGTGGG

>clone2A (117 bp), DLY 1481
TAGGGTAGTGTTAGGGTTGTGGGTGTGTGGGTGTGGTGTGGGTGTGGGTGTGGTGTGGGTGTGGTGTGTGTG
GGTGTGGGTGTGGTGTGTGTGGGTGTGGTGTGTGGGTGTGGGTGTGG

>clone3 (43 bp), DLY 1331
TAGGGTAGTGTTAGGGTTGTGGGTGTGTGGGTGTGGTGTGGGTGTGGGTGTGGTGTGGGTG

>clone4 (152 bp), DLY 1479
TAGGGTAGTGTTAGGGTTGTGGGTGTGTGGGTGTGGTGTGGGTGTGGGTGTGGTGTGGGTGTGGTGTGGGTG
TGGTGTGGGTGTGTGGGTGTGGTGTGTGGGTGTGTGGTGTGTGGGTGTGGGTGTGGTGTGGGTGTGTG
GGTGTGGTGTGGGTGTGGTGTGTGGG

>clone5 (231 bp), DLY 641
TAGGGTAGTGTTAGGGTTGTGGGTGTGTGGGTGTGGTGTGGGTGTGGGTGTGGTGTGGGTGTGGTGTGGGTG
TGGTGTGGGTGTGTGGGTGTGGTGTGTGGGTGTGTGGTGTGTGGGTGTGGGTGTGGTGTGGGTGTGTG
GGTGTGGTGTGGGTGTGGTGTGTGGGTGTGGGTGTGTGTGGGTGTGGTGTGTGGGTGTGTGTGGGTGTGGGT
GTGGTGTGGGTGTGGGTGTGGTGTGGTGTGTGG

>clone6 (234 bp), DLY 640
TAGGGTAGTGTTAGGGTTGTGGGTGTGTGGGTGTGGTGTGGGTGTGGGTGTGGTGTGGGTGTGGTGTGGGTG
TGGTGTGGGTGTGGTGTGTGTGGGTGTGGTGTGTGGGTGTGGGTGTGGTGTGGGTGTGGTGTGGGTGTGG
TGTGGTGTGGGTGTGGTGTGTGGGTGTGGGTGTGGTGTGTGGGTGTGGGTGTGGTGTGGGTGTGGGTG
TGTGGGTGTGGGTGTGGTGTGTGGGTGTGGGTGTGGTGTGTGGGTGTGGGTGTGGTGTGGGTGTGGGTG
TGTGGGTGTGGGTGTGGTGTGTGGGTGTGTGGGTG

Figure 9. Correlation between lengths of telomeric DNA fragments amplified by PCR (ATLAS) and TL determined by TRF (SBH). Linear regression lines show correlation between TRF results obtained by SBH (axis Y) and TL determined by ATLAS (axis X) for chromosomes 1, 6 and 11 in strains with short telomeres (DLY1331 and DLY1481, *mre11*Δ), normal telomeres (DLY640 and DLY641, WT) and long telomeres (DLY5395 and DLY5396, *pif1*Δ *exo1*Δ). The length of telomeric fragments was determined using ladder DNA bands in gels. Six replicas for each strain were used for quantification of TL from SBH, and four to twelve replicas from PCR gels.

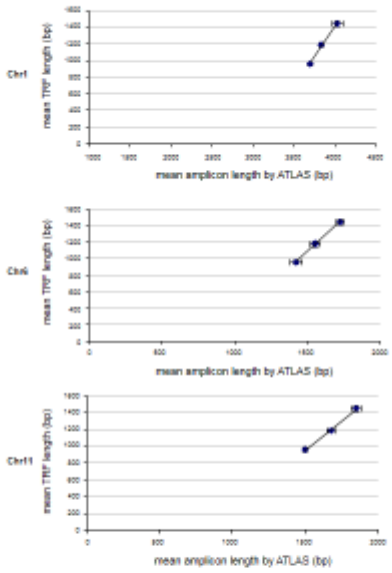

Table 1. Strains of *S. cerevisiae* used in the study.

Strain	Relevant genotype and telomere phenotype	Reference
DLY 640	*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2 RAD5* (wild type); WT telomeres	R. Rothstein
DLY 641	*MATalpha ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2 RAD5* (wild type); WT telomeres	R. Rothstein
DLY 1171	*MATa rif2::HIS3;* long telomeres	D. Lydall
DLY 1296	*MATa cdc13-1int exo1::LEU2;* marginally longer telomeres than in WT	D. Lydall
DLY 1331	*MATalpha mre11::hisG::URA3 RAD5;* short telomeres	[[61]]
DLY 1408	*MATa yku70::HIS3 exo1::LEU2 RAD5;* slightly shorter telomeres than in WT	[[62]]
DLY 1412	*MATa yku70::HIS3 RAD5;* short telomeres	[[62]]
DLY 1479	*MATalpha mre11::hisG::URA3;* short telomeres	D. Lydall
DLY 1481	*MATa mre11::hisG::URA3;* short telomeres	D. Lydall
DLY 5395	*MATa exo1::LEU2 pif1::NATMX;* long telomeres	[[63]]
DLY 5396	*MATalpha exo1::LEU2 pif1::NATMX;* long telomeres	[[63]]
YCR 36	[$RIF1^{+}RIF2^{+}$] (control for YCR 116 and YCR 272); WT telomeres	S. Mattarocci & N.H. Thomä
YCR 116	*rif2::KanMX4;* very long telomeres	[[64]]
YCR 272	*rif1::NatMX4;* very long telomeres	[[65]]

Strains are on W303 background.

Table 2. Chromosome specific and telomeric primers.

Primer name	Primer sequence
Chr.1-F	5'-GGTACGTTCCACAAGGTGCT-3'
Chr.2-F	5'-ACCTCACTGCGGGATAATTG-3'
Chr.6-F1	5'-TAAAGGAATCCCCAGAGACCTC-3'
Chr.6-F2	5'-GCCGAGGACTGTCAATTAGC-3'
Chr.9-F	5'-TAATTTTTGGCCTGCCTCAC-3'
Chr.11-F	5'-AGATGGGGCTAGACGAGACCG-3'
$dG_{(18)}$	5'-GGGGGGGGGGGGGGGGGG-3'
$dG_{(18)}M_{(1)}$ (M=A+C)	5'-GGGGGGGGGGGGGGGGGGM-3'
$dG_{(18)}M_{(2)}$ (M=A+C)	5'-GGGGGGGGGGGGGGGGGG MM-3'
r-ad2	5'-GCGGCCGCTTATTAACCCTCACTAAA-3'

Table 3. PCR conditions for amplification of telomeres from different chromosomes.

Chromosome number	**Primers and enzymes used**	**Initial denaturing**	**Number of cycles***	**Denaturing**	**Annealing**	**Extension**	**Final extension**	**Expected amplicon size****
I	Chr.1-F - $dG_{(18)}$ **[P]**	95°C 2 min	40-45	94°C 30 sec	58°C 30 sec	65°C 4 min	72°C 10 min	3,642 bp
II	Chr.2- F - $dG_{(18)}$ **[P]**	95°C 2 min	40-45	94°C 30 sec	58°C 30 sec	65°C 3 min	72°C 10 min	2,831 bp
VI (short amplicon)	Chr.6-F1 - $dG_{(18)}$ **[P]**	95°C 2 min	40	94°C 20 sec	65°C 30 sec	65°C 1 min 30 sec	72°C 10 min	1,258 bp
VI (long amplicon)	Chr.6-F2 - $dG_{(18)}$ **[P]**	95°C 2 min	40-45	94°C 30 sec	58°C 30 sec	65°C 5 min 30sec	72°C 10 min	5,228 bp
IX	Chr.9-F - $dG_{(18)}$ **[P]**	95°C 2 min	40-45	94°C 30 sec	58°C 30 sec	65°C 4 min 15sec	72°C 10 min	4,082 bp
XI	Chr.11-F - $dG_{(18)}$ **[P]**	95°C 2 min	40	94°C 20 sec	65°C 30 sec	65°C 1 min 30 sec	72°C 10 min	1,500 bp
VI	Chr.6-F1 - r-ad2 (or $dG_{(18)}$) **[Q]**	95°C 15 min	45	94°C 30 sec	62°C 30 sec	72°C 1 min 30sec	72°C 10 min.	1,258 bp
XI	Chr.11-F - r-ad2 (or $dG_{(18)}$) **[Q]**	95°C 15 min	42-45	94°C 30 sec	67°C 30 sec	72°C 1 min 30sec	72°C 10 min.	1,500 bp

[P] - GoTaq® Long PCR Master Mix (Promega); **[Q]** - HotStarTaq® DNA Polymerase (Qiagen).

*45 cycles regime was used also in other studies on telomere PCR [29, 43, 45, 48].

**Calculated according SGD for *S. cerevisiae* with genetic background S288C and does not include the length of the reverse primer.